The Effects of Cations on the Activity of the Gypsy Moth (Lepidoptera: Lymantriidae) Nuclear Polyhedrosis Virus

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ABSTRACT Fourteen cations were tested at a 1% concentration (wt:wt), as chlorides, for their effects on the biological activity of the gypsy moth, *Lymantria dispar* (L.), nuclear polyhedrosis virus (LdMNPV). Cupric chloride was toxic to gypsy moth larvae. Ferrous and ferric chloride were inhibitory to larval growth and development as well as to virus activity. Strontium chloride was inhibitory to virus activity but had no apparent effects on gypsy moth larvae. Six cations had little or no effect on virus activity (i.e., calcium, lanthanum, magnesium, nickel, potassium, sodium), whereas four cations (i.e., cobalt, manganese, ruthenium, zinc) acted as viral enhancers, as indicated by reductions in LC_{50}s.

KEY WORDS *Lymantria dispar*, cations, viral activity, enhancement

SINCE THE DISCOVERY that fluorescent (=optical) brighteners could enhance the biological activity of insect viruses (Hamm and Shapiro 1992; Shapiro and Robertson 1992; Shapiro et al. 1992; Shapiro and Dougherty 1993; Zou and Young 1994, 1996), much effort has been devoted to the mechanism(s) for this enhancement (Adams et al. 1994, Sheppard and Shapiro 1994, Sheppard et al. 1994, Dougherty et al. 1995, Washburn et al. 1998). Currently, the research has provided much valuable data but is primarily in its descriptive stage. The only brighteners that appear to enhance viral activity belong to the class of stilbenes (Shapiro and Dougherty 1993), but not all stilbenes are active (Argauer and Shapiro 1997).

Stilbenes have been studied for many years as inhibitors of human red blood cell exchange (Cabantchik and Rothstein 1972, Rao et al. 1979, Romero and Ortiz 1988, Venglarik et al. 1994, Romero and Boron 1999). These studies have centered on the stilbenes 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and 4-acetamido-4′isothiocyanostilbene-2,2′-disulfonic acid (SITS) and related stilbenes as 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) and demonstrated that both anion and calcium transport were inhibited (Niggli et al. 1982, Romero and Ortiz 1988).

The demonstration that stilbenes are inhibitors of calcium transport, led me to investigate whether organic (Nandi et al. 1990, Sunaga et al. 1990, Cartee et al. 1992) and inorganic (Howell 1982, Sigel et al. 1990, Soler et al. 1992) calcium transport inhibitors could act as viral enhancers. This article reports on the effect of inorganic metals on the activity of the gypsy moth, *Lymantria dispar* (L.), nuclear polyhedrosis virus (LdMNPV).

Materials and Methods

Insects and Virus Inocula. The New Jersey strain of *Lymantria dispar* (L.) (USDA-Aphis, Otis Air National Guard Base, MA) was used and insects were reared on a wheat germ-based diet (Bell et al. 1981). The gypsy moth nuclear polyhedrosis virus inoculum (LdMNPV) was the Hamden isolate LDP-226 (U.S. Forest Service, Hamden, CT).

Bioassays. Polyhedral inclusion bodies (PIBs) were decimally diluted in distilled water to produce concentrations ranging from 10^2 to 10^6 PIBs/ml (=0.02–209.6PIBs/mm^2), and 1 ml of inoculum was pipetted onto the surface in each 180-ml container (=4,770 mm^2; Sweetheart Cup, Chicago, IL). Ten late second instars (7 d old: average weight, 35 ± 6.8 mg per larva) were placed in each container and were maintained for 21 d at 29°C, 50% RH, and a photoperiod of 12:12 (L:D) h. Tests involving LdMNPV and LdMNPV/cation (1% wt:wt) combinations were repeated five times with 30 larvae per virus dilution per treatment per replicate, 30 untreated larvae per replicate, and 30 larvae per cation treatment per replicate. In a second series of tests, cations that appeared to act as virus enhancers were tested at 0.001, 0.01, 0.10, and 1.00% (wt:wt). These tests were repeated four times.

Cations. The cations used were calcium chloride (CAS #10035-04-8), cobalt chloride (CAS #7791-13-1), cupric chloride (CAS #10125-13-0), ferric chloride (CAS #10025-77-1), ferrous chloride (CAS #13478-10-9), lanthanum chloride (CAS #10025-84-0), magnesium chloride (CAS #7791-20-0), manganese chloride (CAS #13446-34-9), nickel chloride (CAS #7791-20-0), potassium chloride (CAS #7447-40-7), ruthenium red (=ammoniated ruthenium oxychloride; CAS #11103-72-3), sodium chloride (CAS #7647-14-5), strontium chloride (CAS #10025-70-4),

Table 1. Effects of cations on the activity of *Lymantria dispar* nuclearpolyhedrosis virus (LdMNPV) activity: LC_{50}

Treatment[a]	LC_{50} (as PIBs/mm^2)[b] (95% CL)	Slope (±SEM)	Activity ratio[c]
LdMNPV alone	9.79 (8.36–11.45)	1.46 (0.01)	1.00
LdMNPV/Monovalent cations			
Potassium	8.36 (6.65–10.55)	1.44 (0.01)	1.17
Sodium	14.03 (10.94–18.01)	1.55 (0.01)	0.71
LdMNPV/Divalent cations			
Calcium	11.64 (9.41–14.38)	1.71 (0.02)	0.82
Cobalt	0.51 (9.41–14.38)	1.30 (0.02)	19.14
Copper	Toxic to larvae		
Iron	>210		<0.05
Magnesium	12.54 (10.29–15.24)	1.66 (0.01)	0.78
Manganese	5.14 (4.09–6.46)	1.06 (0.01)	1.91
Nickel	7.65 (6.25–17.78)	1.22 (0.02)	1.28
Strontium	17.80 (14.21–22.31)	1.53 (0.01)	0.55
Zinc	0.44 (0.34–0.59)	1.05 (0.01)	22.24
LdMNPV/Trivalent cations			
Iron	>210		<0.05
Lanthanum	12.22 (5.01–25.93)	1.67 (0.05)	0.80
Ruthenium	0.05 (0.01–0.23)	1.33 (0.04)	183.14

[a] All cations were tested as chlorides; ruthenium was used as the ammoniated oxychloride (ruthenium red). Only cupric chloride was toxic to gypsy moth larvae.

[b] LC_{50}s were expressed as PIBs/mm^2 of surface area; five replicates; five virus concentrations per treatment per replicate; total = 750 larvae per treatment. Gypsy moth NPV (LdMNPV) was diluted either in distilled water (LdMNPV alone) or in aqueous cation solutions (LdMNPV/cation).

[c] Activity ratio is calculated by dividing the LC_{50} for LdMNPV by LC_{50}s for LdMNPV/cation combinations.

and zinc chloride (CAS #7646-85-7) (Sigma, St. Louis, MO). These chemicals were chosen to represent monovalent, bivalent, and trivalent cations and all were obtained as chlorides. In initial tests, all chemicals were used at 1% (wt:wt). In a second series of tests, cobalt, ruthenium red, and zinc were used at 0.001, 0.01, 0.10, and 1.00% (wt:wt).

Statistical Methods. Concentration-mortality and time-mortality regressions were estimated by probit analysis (LeOra Software 1987) to monitor the biological activities of LdMNPV with and without salts. Failure of 95% CL was used as a criterion for significant differences.

Results

Fourteen cations (as chlorides) were tested as enhancers for LdMNPV (Table 1). One cation (i.e., cupric chloride) was toxic to gypsy moth larvae at a 1% concentration and was not tested further. Both ferrous and ferric chloride had detrimental effects on gypsy moth larvae (i.e., stunted) and gypsy moth NPV activity (i.e., greatly increased LC_{50}s). For both iron compounds, LC_{50}s could only be obtained in one replicate and in one replicate no virus-caused mortality was obtained at a concentration of 210 PIBs/mm^2 ($=10^6$ PIBs per container). None of the other cations appeared to have detrimental effects on gypsy moth larvae at the concentration used. Strontium appeared to be the only other cation that inhibited viral activity (Table 1). Potassium, sodium, calcium, magnesium, nickel, and lanthanum had little effect on virus activity. Manganese (twofold), cobalt (19-fold), zinc (22-fold), and ruthenium (180-fold), however, reduced LC_{50}s (Table 1). The addition of cobalt and ruthenium also decreased the speed of kill (i.e., LT_{50}); whereas, the addition of lanthanum and magnesium increased the LT_{50}s (Table 2). The remaining cations, with the exceptions of copper and iron, appeared to have little effect on the speed of kill (Table 2).

Following this test, a subsequent study was set up to investigate the relationship between cation concentration and virus enhancement, using cobalt, ruthenium, and zinc (Table 3). In general, enhancement activities of these cations increased as the concentra-

Table 2. Effect of cations on LdMNPV activity: LT_{50}

Treatment[a]	LT_{50} (95% CL) in days[b]	Relative speed of kill, %[c]
LdMNPV alone	10.9 (10.3–11.5)	—
LdMNPV/Monovalent cation		
Potassium	11.4 (10.8–12.1)	+4.5
Sodium	11.1 (10.3–11.8)	+1.8
LdMNPV/Divalent cation		
Calcium	11.5 (10.9–12.1)	+5.5
Cobalt	9.6 (9.0–10.0)	−11.9
Copper	Toxic to larvae	
Iron	—[d]	
Magnesium	12.3 (11.6–13.0)	+12.8
Manganese	11.7 (10.9–12.5)	+7.3
Nickel	12.1 (11.3–12.9)	+11.0
Strontium	11.6 (11.3–12.3)	+6.4
Zinc	10.8 (10.2–11.5)	−1.0
LdMNPV/Trivalent cation		
Iron	—[d]	
Lanthanum	12.1 (11.6–12.8)	+11.0
Ruthenium	8.9 (8.5–9.4)	−18.3

[a] LdMNPV was diluted in distilled water (LdMNPV alone) or in aqueous cation solutions (LdMNPV/cation).

[b] LT_{50}s were determined for a virus concentration of 210 PIBs/mm^2 of surface area and were averaged for five replicates per treatment.

[c] The LT_{50} for LdMNPV alone was used as the standard and all other LT_{50}s were compared to the standard. Negative values indicate that the treatment resulted in faster kill than the standard. Positive values indicate that the treatment resulted in slower kill than the standard.

[d] LT_{50} values could not be determined at a virus concentration of 210 PIBs/mm^2.

Table 3. Effect of different concentrations of cobalt chloride, ruthenium red, and zinc chloride on LdMNPV activity: LC_{50}

Treatment[a]	Cation concn	LC_{50} (PIBs per mm^2)[b] (95% CL)	Slope (±SEM)	Activity ratio[c]
LdMNPV alone	0	8.79 (6.35–12.49)	1.62 (0.01)	1.00
LdMNPV/Cobalt	0.001	7.32 (6.65–10.44)	1.39 (0.01)	1.20
	0.010	4.42 (3.65–5.41)	2.16 (0.03)	1.99
	0.100	2.70 (2.21–3.33)	1.97 (0.02)	3.25
	1.000	0.32 (0.25–0.40)	1.59 (0.01)	27.41
LdMNPV/Ruthenium	0.001	8.07 (6.52–9.96)	2.17 (0.04)	1.09
	0.010	3.90 (3.10–4.91)	1.85 (0.02)	2.25
	0.100	0.22 (0.10–0.50)	1.92 (0.02)	39.94
	1.000	0.03 (0.02–0.08)	1.33 (0.02)	262.13
LdMNPV/Zinc	0.001	13.65 (10.80–16.90)	1.79 (0.03)	0.64
	0.010	5.09 (4.21–6.23)	2.13 (0.05)	1.73
	0.100	3.23 (2.68–3.88)	2.21 (0.05)	2.72
	1.000	0.37 (0.24–0.50)	1.48 (0.02)	23.97

[a] LdMNPV was diluted in distilled water (LdMNPV alone) or in an aqueous cation solution (0.001, 0.010, 0.100, 1.00% wt:wt). No larval mortality was observed among larvae exposed to any of the cations.
[b] LC_{50}s are expressed as PIBs per mm^2; four replicates; five virus concentrations per treatment per replicate; total = 600 larvae per treatment.
[c] Activity ratios are calculated by dividing the LC_{50} for LdMNPV alone by LC_{50}s for each LdMNPV/cation combination.

tion of the cations increased. In the case of cobalt chloride and zinc chloride, the greatest incremental increase in enhancement occurred as the concentration increased from 0.10 to 1.00% (e.g., eight- to ninefold). For ruthenium red, the greatest increase in enhancement activity (e.g., 18-fold) occurred as the concentration increased from 0.10 to 1.00%. A further sevenfold increase occurred with an increase in ruthenium red concentration to 1.00%, resulting in a total 260-fold increase in LdMNPV activity (Table 3).

The amount of virus required to produce an LC_{10}, LC_{30}, LC_{50}, LC_{70}, and LC_{90} combinations of LdMNPV/cobalt, LdMNPV/ruthenium, or LdMNPV/zinc was significantly less ($P < 0.05$) than that required for LdMNPV at every point in the mortality curve (Table 4). Whereas, 8.4 PIBs/mm^2 were required to produce 50% mortality among LdMNPV-treated larvae, only 0.38, 0.34, and .04 PIBs/mm^2 were required to achieve the same mortality level among LdMNPV/zinc-, LdMNPV/cobalt-, and LdMNPV/ruthenium-treated larvae, respectively. More than 42 PIBs/mm^2 were required to achieve 90% kill among LdMNPV-treated larvae, whereas only 0.31–2.73 PIBs/mm^2 were required in the three LdMNPV-cation treatments. At every mortality level, less NPV was needed in the LdMNPV/ruthenium treatment than in any other treatment. In these treatments (e.g., LdMNPV only, LdMNPV/cobalt, LdMNPV/ruthenium, LdMNPV/zinc) the amount of NPV to increase mortality from 10 to 50% was similar to that required to increase mortality from 50 to 90%. For example, sixfold more NPV was needed to increase virus kill from 10 to 50%, and a further sixfold increase in NPV was needed to increase virus-caused mortality from 50 to 90% among LdMNPV only and LdMNPV/cobalt treatments. Thus, the addition of these cations to LdMNPV suspensions resulted in significant differences in LC_{50} and LC_{90} among treatments but did not change the ratios of LC_{90} to LC_{50} within a given treatment.

Discussion

Little is known about the effect of cations on viral activity. More than 40 yr ago, research was conducted in Europe and Japan on the induction of virus diseases (Vago 1951, Yamafuji 1952, Aruga 1958, Steinhaus 1958) but the results were often contradictory. Subsequent research on the effects of chemical stressors on virus incidence led to both negative (Bird 1955, Steinhaus 1858, Shapiro 1961) and positive (Yadava 1971, Shapiro and Bell 1982) results, depending on the insect species and the chemical used. In the 1970s and 1980s some effort was made to explain inactivation of NPVs on cotton leaves. McLeod et al. (1977) noted that cotton dew had a pH above 10 and implicated magnesium ions in cotton dew as responsible for the inactivation of *Helicoverpa* (*Heliothis*) *zea* (Boddie)

Table 4. Comparison of LC values for LdMNPV alone and LdMNPV/cobalt chloride (1%), LdMNPV/ruthenium red (1%), and LdMNPV/zinc chloride (1%) combinations

Treatment	LC values (95% CL) as PIBs/mm^2[a]				
	LC_{10}	LC_{30}	LC_{50}	LC_{70}	LC_{90}
LdMNPV alone	1.43 (1.11–1.74)	4.17 (3.53–4.86)	8.79 (7.59–10.17)	18.53 (15.87–34.40)	54.54 (44.23–69.43)
LdMNPV/Cobalt	0.05 (0.03–0.07)	0.15 (0.12–0.19)	0.32 (0.25–0.40)	0.69 (0.53–0.88)	2.05 (1.45–2.91)
LdMNPV/Ruthenium	0.004 (0.001–0.006)	0.014 (0.01–0.02)	0.03 (0.02–0.08)	0.08 (0.04–0.13)	0.31 (0.14–0.69)
LdMNPV/Zinc	0.05 (0.03–0.08)	0.21 (0.14–0.40)	0.37 (0.24–0.50)	0.83 (0.52–1.05)	2.70 (1.79–3.35)

[a] LdMNPV was diluted in distilled water (LdMNPV alone) or in aqueous cation solutions (1%). Table 4 represents an expansion of Table 3 to include several LC values.

nuclear polyhedrosis virus HzSNPV. Young et al. (1977) observed that viral inactivation of cotton was much greater than on soybean leaves and that greater amounts of Mg^{2+} were found in cotton dew than in soybean dew. Elleman and Entwistle (1982, 1985a) noted that the NPV from *Spodoptera littoralis* (Boisduval) (SlMNPV) was also inactivated on cotton leaves. Whereas divalent cations in leaf gland exudates were shown to decrease the solubility of PIBs, Mg^{2+} alone was not responsible for the inactivation of the virus (Elleman and Entwistle 1985b).

In the case of the gypsy moth, cupric chloride (1%) was toxic and was not considered further. Both ferrous and ferric chloride were inhibitory to larval growth and development, as well as to the gypsy moth NPV at the 1% concentration. At concentrations of 0.001, 0.01, and 0.10% little or no adverse effects were observed. Strontium chloride (at 1%) had no adverse effect on larvae but appeared to inhibit NPV, as evidenced by an increase in the LC_{50} (Table 1). The great majority of cations, (i.e., monovalent catiopns as potassium, sodium; divalent cations as calcium, magnesium, and nickel); and trivalent cations as lanthanum), however, had little or no effect (at 1%) on gypsy moth larvae or NPV-caused mortality.

At a 1% concentration, three divalent (cobalt, manganese, and zinc) and one trivalent (ruthenium) cation decreased LC_{50}s of LdMNPV. Manganese enhanced activity by twofold, cobalt and zinc by 20-fold, and ruthenium by 260-fold (Table 1). In all cases, no enhancement occurred at a concentration of 0.001% but became significant at 0.01% (Table 3). In the case of both cobalt and zinc chloride, the greatest increase (i.e., eight- to ninefold) occurred as the concentration was increased from 0.10 to 1.00%. In the case of ruthenium red, the greatest increase (i.e., 18-fold) occurred at lower concentrations (i.e., when the concentration was increased from 0.01 to 0.10%). A further sevenfold increase occurred as the concentration was increased from 0.10 to 1.00%, resulting in a total 260-fold enhancement. In the case of both cobalt and zinc, LC values were reduced by 20- to 30-fold (in comparison to that of LdMNPV alone) at every value from LC_{10} to LC_{90}. In the case of ruthenium red, the greatest effect of ruthenium red (1%) occurred at the LC_{10}, where the value for MNPV/ruthenium red was 380-fold less than that for LdMNPV alone (Table 4). At subsequent LC values (LC_{30}, LC_{50}, LC_{70}, LC_{90}) the differences were reduced but were still highly significant ($P < 0.01$). The effects of these cations on the speed of virus-caused mortality (LT_{50}) were less than those of virus concentration (LC_{50}) (Table 2). The addition of zinc chloride to LdMNPV did not change to LT_{50}, whereas the LT_{50}s were reduced by 1.3 d (=12%), and by 2.0 d (=18%) by the addition of cobalt chloride and ruthenium red, respectively. Using both the lethal concentration (LC_{50}) and the speed of kill (LT_{50}) as criteria of enhancement, ruthenium red appeared to be the most active cation tested.

Although no tests were conducted to investigate the possible mode(s) of action of these enhancing cations, some clues do exist. Several of the cations tested have been shown to act as calcium channel blockers in different plant and animal systems. Lanthanum (Richard et al. 1990, Schiffman et al. 1995), magnesium (Meissner et al. 1986, Hattori and Maehashi 1994), and nickel (Dryl and Lopatowska 1990, Yamaguchi and Uematsu 1990) have been shown to inhibit calcium uptake or efflux but they did not act as enhancers for LdMNPV; whereas, cobalt (Scheenen et al. 1994, Philosoph-Hadas 1996 et al.), manganese (Yamaguchi and Uematso 1990), ruthenium red (Howell 1982, Taipale et al. 1989), and zinc (Kruse and Poppele 1985, Koohmaraie 1992) enhanced the activity of LdMNPV. In addition, cobalt chloride has been used as stain for neurons in insects (Jagota and Habibulla 1992, Brockmann and Brueckner 1995) and ruthenium red has been used as a stain for polysaccharides (Kuno et al. 1986, Mattila 1989).

These results lead us to ask what the relationship between calcium channel inhibition and viral enhancement is. Several cations that do not act as enhancers do act as calcium channel inhibitors (i.e., lanthanum, magnesium, nickel), but these non-effects could be a function of cation concentration. The enhancing cations have certainly been shown to act as calcium channel blockers in diverse biological systems, but does the inhibition or blocking of calcium influx or efflux result in an increase of viral activity in a given insect-virus system? This important question is the subject of a larger research effort to determine the factors affecting pathogenicity of LdMNPV as influenced by stilbene optical brighteners, anion transport inhibitors, cation transport inhibitors, and carbonic anhydrase inhibitors in the gypsy moth-LdMNPV system.

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